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Expression of lactoferrin receptors is increased in the mesencephalon of patients with Parkinson disease

(neurodegenerative disease/iron/cell death)

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ABSTRACT **The degeneration of nigral dopaminergic neurons in Parkinson disease is believed to be associated with oxidative stress. Since iron levels are increased in the substantia nigra of parkinsonian patients and this metal catalyzes the formation of free radicals, it may be involved in the mechanisms of nerve cell death. The cause of nigral iron increase is not understood. Iron acquisition by neurons may occur from iron–transferrin complexes with a direct interaction with specific membrane receptors, but recent results have shown a low density of transferrin receptors in the substantia nigra. To investigate whether neuronal death in Parkinson disease may be associated with changes in a pathway supplementary to that of transferrin, lactoferrin (lactotransferrin) receptor expression was studied in the mesencephalon. In this report we present evidence from immunohistochemical staining of postmortem human brain tissue that lactoferrin receptors are localized on neurons (perikarya, dendrites, axons), cerebral microvasculature, and, in some cases, glial cells. In parkinsonian patients, lactoferrin receptor immunoreactivity on neurons and microvessels was increased and more pronounced in those regions of the mesencephalon where the loss of dopaminergic neurons is severe. Moreover, in the substantia nigra, the intensity of immunoreactivity on neurons and microvessels was higher for patients with higher nigral dopaminergic loss. These data suggest that lactoferrin receptors on vulnerable neurons may increase intraneuronal iron levels and contribute to the degeneration of nigral dopaminergic neurons in Parkinson disease.**

The mechanism of cell death in neurodegenerative disorders is unknown. One of several hypotheses is that the production of free radicals could be increased and this might be involved in the degeneration of nigral dopaminergic (DA) neurons that occurs in Parkinson disease (PD) (1, 2). Reactive oxygen species derived from oxygen are capable of damaging biological molecules, including lipids, carbohydrates, proteins, and nucleic acids (1–4). Excessive amounts of oxyradicals can be formed when transition metal ions, especially iron, interact with hydrogen peroxide to form the highly reactive hydroxyl radical. Iron has been measured throughout the brain, with highest concentration in the basal ganglia, especially the globus pallidum and substantia nigra (5–7). Excess levels have been demonstrated in the substantia nigra pars compacta (SNpc) of patients dying with PD and melanized neurons accumulate this metal during PD (4, 8–11). The excessive accumulation of iron in the SN of PD patients, together with changes in superoxide dismutase activity, reduced glutathione, and decreased or unchanged ferritin, is compatible with the concept of an active toxic process involving reactive oxygen species (1, 2, 10). The cause of high iron levels is unknown and may result from increased uptake or decreased release.

Among various pathways to penetrate into cells, iron can be taken up from transferrin in a receptor-mediated process (12). However, two regional analyses of radiolabeled ferrotransferrin binding sites in the mesencephalon have shown that their density was extremely low in the SNpc of controls and was either unchanged or decreased in PD patients (13, 14), which indicates that the increase in nigral iron content reported in surviving neurons (4, 8, 11) and in glial cells (7) may occur (*i*) by metabolic changes with a higher penetration of iron without any increased density of transferrin receptors on the soma of DA cells, (*ii*) by receptors located on other parts of DA neurons (i.e., terminals), and/or (*iii*) by other pathways. A localization of transferrin receptors on DA terminals in the striatum has been suggested by studies of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (15) or MPTP-treated monkeys (16) and human brains taken at postmortem (15, 16), but this still needs to be confirmed. The receptors of lactoferrin (previously also called lactotransferrin; Lf) (17) could play a role in iron uptake by neurons, since Lf expression has recently been observed in postmortem brain tissues of normal controls and patients with neurodegenerative diseases (18, 19). Characterization and isolation of the receptor for Lf have been performed for various cell types in humans (20–25). The Lf receptor is a monomeric glycoprotein of 105 kDa (20, 24), while the transferrin receptor is a disulfide-linked homodimer of 180 kDa with two transferrin binding sites (26). In the present study we analyzed the expression of Lf receptors in the SNpc of controls and PD patients, using polyclonal antibodies directed against the human Lf receptor and immunohistochemical techniques, in order to determine whether the presence of this protein could explain increased nigral iron levels in PD.

MATERIALS AND METHODS

Human Brain Tissue. Mesencephalons were obtained at autopsy from 13 individuals with no known history of psychiatric or neurological disorders and from 8 patients with histologically confirmed PD. All patients had a clinical history compatible with the diagnosis of PD and were responsive to L-dopa treatment. They were judged to have moderate to

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Abbreviations: DA, dopaminergic; Lf, lactoferrin; PD, Parkinson disease; SNpc, substantia nigra pars compacta; AU, arbitrary units.

severe illness, with marked bradykinesia, rigidity, and tremor [stages III–V on the Hoehn–Yahr rating scale (27)]. Age at death and time interval from death to tissue fixation did not differ significantly between the group of controls [80 ± 3 yr and 18 ± 2 hr, respectively (mean ± SEM)] and the group of PD patients (81 ± 2 yr and 26 ± 3 hr, respectively). Within 2 hr of autopsy, tissue was fixed for 72 hr in 4% paraformaldehyde/15% picric acid in 0.1 M phosphate-buffered saline solution (pH 7.4) and processed as described (28).

Immunohistochemistry. Free-floating 40-μm-thick sections (at the level of the oculomotor nerve fibers) were incubated in the presence of an IgG fraction of polyclonal rabbit antibodies (1:1000, in 0.25 M Tris·HCl-buffered saline at pH 7.4; 72-hr incubation at 4°C) raised against the Lf receptor previously isolated from human platelet lysates by affinity chromatography on immobilized mouse monoclonal antibody DP5B3G10 raised against the soluble Lf binding protein purified from the cell culture supernatant of a lymphoblastic Jurkat T-cell line (29). The sections were immunostained by the avidin-biotin horseradish peroxidase complex procedure with 3,3′-diaminobenzidine as a cosubstrate (28).

Sections, regularly spaced (1440 μm apart) between the rostral and caudal poles of the SN, were immunostained by the same procedure to identify nigral DA neurons in each mesencephalon, using a mouse monoclonal antibody directed against tyrosine hydroxylase (Incstar, Stillwater, MN; 1:250 dilution; 48-hr incubation at 4°C).

Immunoblots. Homogenates of SNpc or caudate nucleus from a control human subject and of Jurkat leukemia T-cell line were fractionated, and the proteins were separated by polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked in the presence of 5% nonfat milk, incubated overnight at 4°C with the polyclonal antibodies directed against Lf receptors, and the reaction was visualized by the avidin-biotinylated peroxidase complex method with 3,3′-diaminobenzidine as a cosubstrate.

Acetylcholinesterase Histochemistry. Sections adjacent to those treated for immunohistochemistry were stained for acetylcholinesterase activity (30) in order to delineate accurately five anatomical regions: ventrolateral part of the SNpc, dorsal part of the SNpc, ventral tegmental area, peri- and retrorubral area (catecholaminergic cell group A8), and nucleus of the oculomotor nerve (28, 31).

Regional Quantifications and Image Analysis. Measurements of Lf receptor immunoreactivity intensity on stained neurons and microvessels were performed by two independent observers (E.C.H. and P.D.) using a Leitz Laborlux D microscope connected to a charge-coupled device color camera (DXC-101P; Sony) through a Leitz zoom set at 12.5× and a 0.1× tube adaptor. Fields of interest and cells were observed on a color video monitor. A 25× objective was used to observe neurons and microvessels, and a 40× objective was used for glial cells. The intensity of Lf receptor-like immunoreactivity on neurons and microvessels was estimated using a rating scale ranging from 0 to 4 arbitrary units (AU). Sections were presented in randomized order and the two observers were unaware of the origin of the sections. Their quantifications were very similar and highly correlated (neurons: $r = 0.93$, $P < 0.001$; microvessels: $r = 0.98$, $P < 0.001$). Each regional change of Lf receptor expression level was calculated as the difference between the level measured in PD patients and that measured in controls and expressed as a percentage of the level of controls. The total numbers of neurons stained for tyrosine hydroxylase and located in the SNpc were independently estimated for each brain, using a computer-assisted image analysis system (HistoRag, Biocom, Les Ulis, France). Neurons were plotted and counted for each series of rostrocaudal sections. For each midbrain, the total number of neurons was calculated by integration (28).

Statistical Analyses. Data are presented as the mean ± SEM. Means of the studied variables were compared by Student's t test or two-way ANOVA. Calculations were performed using BMDP STATISTICAL Software (32). The null hypothesis was rejected for an α risk equal to 5%.

RESULTS

Specificity of Antibodies Directed Against Human Lf Receptor. The Lf receptor antibodies used in these experiments were characterized by Western immunoblotting of proteins extracted from human SNpc, caudate nucleus, and human T-lymphoblastic cell line Jurkat homogenates: a single band corresponding to 105 kDa was observed (Fig. 1); no crossreactivity was observed with antibodies directed against the transferrin receptor, for which a single band corresponded to 180 kDa. On tissue sections, omission of the primary antibodies resulted in an absence of labeling, and dilution of the antibodies from 1:100 to 1:50,000 resulted in a progressive decrease of the staining (data not shown). Preadsorption tests could not be performed because purified Lf receptor was not available in sufficient amounts.

Expression of Lf Receptors in Control Subjects. At the macroscopical level, Lf receptor immunoreactivity was observed in the SNpc, the ventral tegmental area, the red nucleus, the perirubral region (catecholaminergic cell group A8), the periaqueductal gray substance, the nucleus of the oculomotor nerve, and the superior colliculus. Staining intensity was much lower in regions containing bundles of fibers, such as the cerebral peduncle and the lemniscus medialis. At the microscopical level, moderate to high Lf receptor immunoreactivity was observed in the perikarya, apical dendrites and axons of neurons in the SNpc (Figs. 2 and 3), the ventral tegmental area, the nucleus of the oculomotor nerve, and the periaqueductal gray substance. The presence of neuromelanin in some stained neurons suggests that some of them were DA neurons. Lf

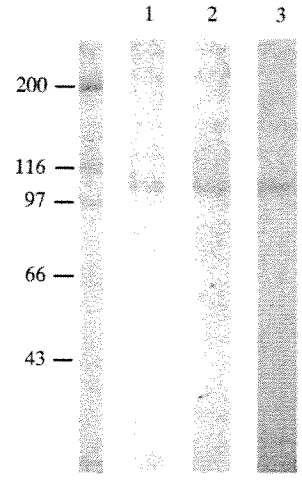

FIG. 1. Immunoblot analysis showing expression of Lf receptor protein in two regions of normal human brain and in T lymphocytes (positive control). Molecular mass markers in the first lane are given in kDa. Lanes: 1, homogenate of SNpc; 2, homogenate of caudate nucleus; 3, homogenate of cultured T lymphocytes (Jurkat leukemia T-cell line). Samples (lane 1, 20 μg of protein; lane 2, 15 μg of protein; lane 3, 10^7 cells) were electrophoresed on a single 7.5% acrylamide slab gel containing 0.1% SDS, transferred to nitrocellulose membranes, and immunostained with rabbit polyclonal antibodies directed against the human Lf receptor (lanes 1 and 2, 1:1000; lane 3, 1:2500). Only one band is seen and recognized at an apparent molecular mass of about 105 kDa. Diminished immunoreactivity of lane 1 as compared to lane 2 is not the result of reduced protein load and corresponds to tissue differences also observed by immunohistochemical controls on tissue sections.

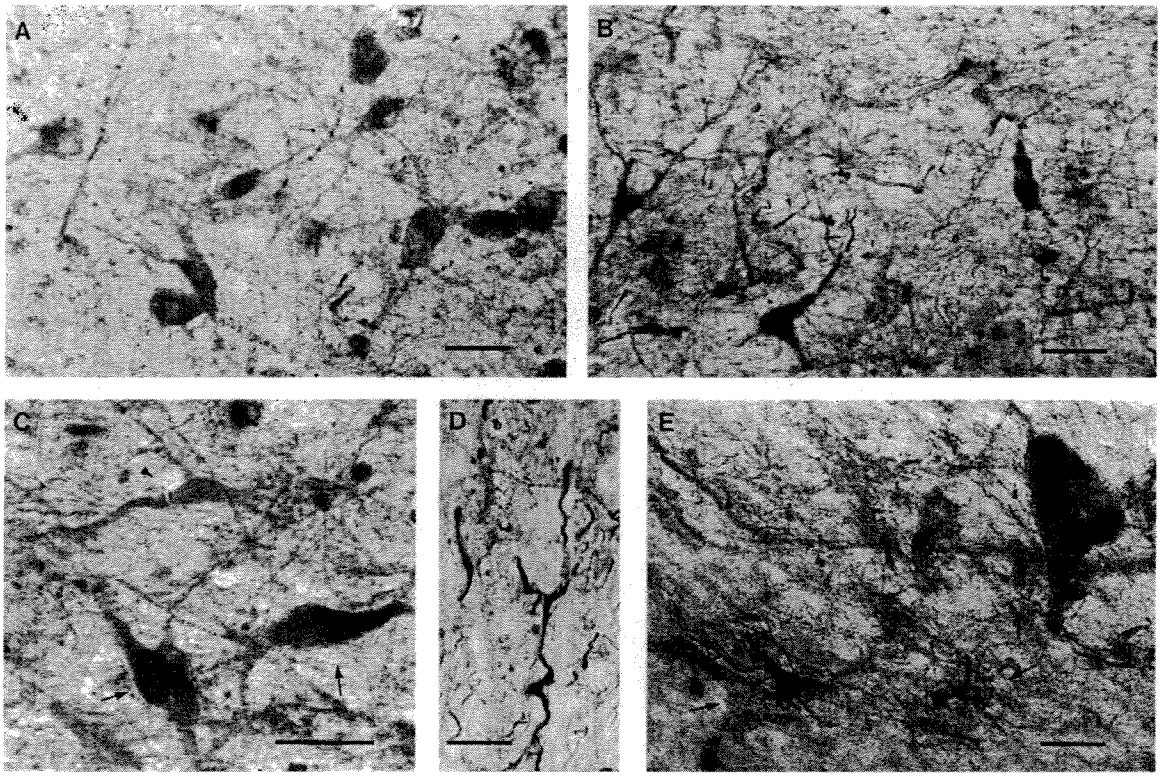

FIG. 2. Immunostaining with rabbit polyclonal antibodies directed against the human Lf receptor on transverse sections of the mesencephalons of control subjects (*A* and *C*) and patients with PD (*B, D,* and *E*). (*A, B,* and *D*) Low-power views of the SNpc to illustrate Lf receptor expression on neurons (*A* and *B*) and blood microvessels (*D*). (*C* and *E*) High-power views of the SNpc showing Lf receptor-positive neurons containing neuromelanin (arrows) or containing no pigment (arrowhead) in a section from a control subject (*C*) and glial cells in the vicinity of a neuromelanized neuron in a section from a parkinsonian patient (*E*). (Bars: *A–D,* 50 μm; *E,* 20 μm.)

receptor immunoreactivity was also detectable on microvessels, though with marked interindividual differences in the level of labeling intensity. Immunopositive blood cells in blood vessels were observed. Some Lf receptor-immunoreactive microvessels were in close proximity to melanized neurons in the SNpc and ventral tegmental area. Labeling was observed on glial cells in sections from two control subjects. Their morphology and size suggest that most of these cells were astrocytes.

Expression of Lf Receptors in Parkinsonian Patients. Stronger immunostaining was observed in PD patients than in controls (Figs. 2 and 3). Staining intensity of neurons was increased in the DA regions affected in PD: ventrolateral SNpc, +110%; dorsal SNpc, +167%; ventral tegmental area, +44%; area A8, +96%. Lf receptor immunoreactivity on neurons and microvessels measured in the nuclei of the third cranial nerve was unchanged. The labeling on microvessels was also increased (Fig. 3): ventrolateral SNpc, +104%; dorsal SNpc, +87%; ventral tegmental area, +41%; area A8, +95%. Mean levels measured for PD patients were significantly higher than those of controls (Fig. 3). Some Lf receptor-immunoreactive glial cells were also observed in PD patients. In three of the eight PD patients, the staining was intense (Fig. 2*E*).

Correlations Between Neurons and Microvessels. There was a significant positive correlation between the Lf receptor immunoreactivity intensity measured on neurons and that on microvessels located in the SNpc or the ventral tegmental area, both for controls (ventrolateral SNpc: $r = 0.55, P < 0.05$; dorsal SNpc: $r = 0.58, P < 0.05$; VTA: $r = 0.57, P < 0.05$) and for PD patients (ventrolateral SNpc: $r = 0.89, P < 0.01$; dorsal SNpc: $r = 0.66$, not significant; VTA: $r = 0.75, P < 0.05$).

In the ventrolateral SNpc, the lower the number of DA nigral neurons the higher the Lf receptor immunoreactivity intensity measured on neurons or on microvessels (Fig. 4). The slopes of the corresponding regression lines tended to be steeper in PD patients than in controls: the more severe the neuronal loss the higher the Lf receptor expression (neurons: −1.6 AU per 10^5 DA neurons vs. −0.6 AU per 10^5 DA neurons, respectively; microvessels: −1.8 AU per 10^5 DA neurons vs. −0.6 AU per 10^5 DA neurons, respectively). Conversely, for the dorsal part of the SNpc, no such trend was observed, either in PD patients or in controls. There was a tendency for Lf receptor expression on ventrolateral SNpc neurons to be negatively correlated with DA neuron counts in the SNpc of PD patients ($r = -0.67, P = 0.07$) and, to a lesser degree, of controls ($r = -0.30, P = 0.31$). This correlation was highly significant when the two groups were combined ($r = -0.73, P < 0.001$; Fig. 4).

DISCUSSION

We observed Lf receptor immunoreactivity on neurons, cerebral microvasculature, and glial cells in the human mesencephalon. The specificity of the antibodies to human Lf receptor was demonstrated by a single band corresponding to 105 kDa observed on Western immunoblotting of proteins extracted from human SNpc, in agreement with reports on Lf receptor characterization (20, 24), and by control investigations on tissue sections (see *Results*). The expression of Lf receptors was increased on the neurons of the SNpc or ventral tegmental area and area A8, where about 80% or 50% of DA neuronal loss occurs (28, 31), whereas there was no significant change in the oculomotor nucleus, where no neuronal loss has been reported in PD. These data suggest a relationship between the loss of

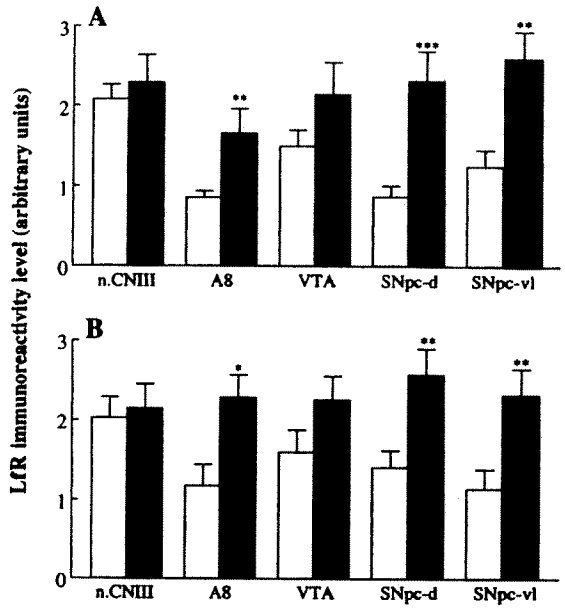

FIG. 3. Comparative mean levels of intensity of human Lf receptor expression (LfR; AU, 0–4 rating scale) in neurons (*A*) and blood microvessels (*B*) of control subjects (n = 13, open bars) and parkinsonian patients (n = 8, solid bars) measured in the indicated anatomical regions of the mesencephalon. A8, perirubral catecholaminergic cell group A8; n.CNIII, nucleus of the oculomotor nerve; SNpc-d, dorsal part of the SNpc; SNpc-vl, ventrolateral part of the SNpc; VTA, ventral tegmental area. Each value represents mean ± SEM. The groups of controls and parkinsonian patients differed in Lf receptor expression levels, according to two-way ANOVA taking into account the region factor and the pathology factor [neurons: $F(1, 17) = 16.68$, $P < 0.001$; microvessels: $F(1, 16) = 5.41$, $P = 0.03$]. Significant differences between parkinsonian patients and control subjects by anatomical region, according to Student's t test: *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.001$.

DA neurons and the increased expression of Lf receptors. Moreover, the changes in Lf receptor protein content do not reflect a general overexpression of metabolic processes in surviving nigral DA neurons, since a decrease in the cellular content of tyrosine hydroxylase protein and messenger RNA has previously been described in the same neuronal population (33).

The expression of Lf receptor was increased in the surviving neurons of PD patients, especially in the SNpc, where iron

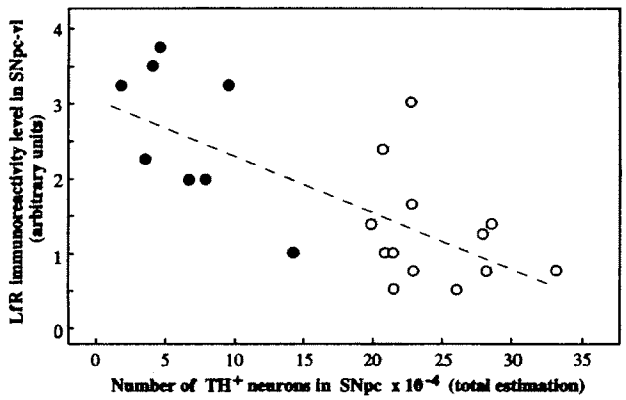

FIG. 4. Lf receptor expression level (LfR; AU) measured in neurons of the ventrolateral part of the SNpc (SNpc-vl) as a function of the number of tyrosine hydroxylase-positive (TH^+) neurons estimated in the SNpc of control subjects (n = 13, ○) and parkinsonian patients (n = 8, ●).

accumulates in the disease (1–4, 7–11, 34), suggesting that iron may enter neurons through these receptors. Although there has been no evidence up to now that iron-loaded Lf is present in brain extracellular fluid, this possibility may be considered as Lf has been measured in the cerebrospinal fluid (35). Glial cells, which also contain iron in the SNpc of PD patients (7), were also stained in sections from three patients (Fig. 2), and an expression of Lf receptor was also observed on brain microvessels of control and PD patients. Accordingly, uptake by perivascular cells may occur for Lf and iron penetration through the blood–brain barrier, a possible pathway parallel to that of transferrin receptors for iron-loaded transferrin to enter the brain compartment (36). A route with Lf receptor located on vascular endothelial cells within the brain is compatible with investigations by positron emission tomography showing that uptake of [^{52}Fe]iron(III) citrate from plasma is increased in the brains of PD patients (37, 38). Thus, Lf receptors may have a role that is complementary to that of the transferrin receptors on the surface of brain capillary endothelial cells (39, 40) to ensure that the brain compartment receives some of the iron required for normal function and participate in physiopathological processes.

The mesencephalic region in which we observed the highest increases of Lf receptor expression on neurons in PD patients is the SNpc, the region where the most severe DA neuronal loss takes place (28, 41). Smaller increases occurred in the ventral tegmental area and area A8, two regions where DA neuron vulnerability is lower (28, 41). The parallel between neuronal vulnerability and changes in Lf receptor expression in the regions studied, especially in the ventrolateral part of the SNpc (Fig. 4), where DA losses are greatest (31), is in accordance with the hypothesis of reactive free iron accelerating oxidative stress and contributing to the destruction of pigmented nigral neurons (1–4, 7–11). As a matter of fact, Lf binds iron more avidly than transferrin; however, little is known about the regulation of iron release from Lf (17, 42, 43). Iron has been shown to be transferred intracellularly through Lf receptors in human monocytes (24, 25) and across cells of the human intestine (22, 44). In contrast to the regulation of the expression of messenger RNA coding for the synthesis of the transferrin receptor, which is closely related to intracellular iron concentrations and the messenger RNA coding for the synthesis of ferritin (45), the expression of Lf receptors in some leukemia cell lines has been reported not to be regulated by intracellular iron (46), which may lead to an unregulated excessive uptake of iron bound to Lf.

Iron levels are also elevated in other neurodegenerative disorders in certain brain regions, which indicates that a relationship between iron and oxidant stress contributing to neuronal death is not exclusive of PD (10, 47, 48), and this is also apparently the case for Lf (18, 19). However, although iron accumulates within the SN in a number of movement disorders of the basal ganglia, these neurodegenerative diseases are presumed to be due to different pathologic mechanisms (10). Particular interrelationships between a selective increase of iron and specific characteristics of vulnerable cells probably potentiate oxidative stress and neuronal death in the SNpc of PD patients.

In conclusion, our results indicate (*i*) that Lf receptors may be involved in neuronal iron uptake, especially in the SNpc of parkinsonian patients, where this metal accumulates during the disease, thereby contributing to the degeneration of melanized DA neurons, and (*ii*) that Lf receptor expression on blood–brain vasculature is increased in PD and may be involved in changes of the blood–brain barrier in this disease.

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